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Novel mutations and mutation combinations of ryanodine receptor in a chlorantraniliprole resistant population of *Plutella xylostella* (L.)

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A previous study documented a glycine to glutamic acid mutation (G4946E) in ryanodine receptor (RyR) was highly correlated to diamide insecticide resistance in field populations of *Plutella xylostella* (Lepidoptera: Plutellidae). In this study, a field population collected in Yunnan province, China, exhibited a 2128-fold resistance to chlorantraniliprole. Sequence comparison between resistant and susceptible *P. xylostella* revealed three novel mutations including a glutamic acid to valine substitution (E1338D), a glutamine to leucine substitution (Q4594L) and an isoleucine to methionine substitution (I4790M) in highly conserved regions of RyR. Frequency analysis of all four mutations in this field population showed that the three new mutations showed a high frequency of 100%, while the G4946E had a frequency of 20%. Furthermore, the florescent ligand binding assay revealed that the RyR containing multiple mutations displayed a significantly lower affinity to the chlorantraniliprole. The combined results suggested that the co-existence of different combinations of the four mutations was involved in the chlorantraniliprole resistance. An allele-specific PCR based method was developed for the diagnosis of the four mutations in the field populations of *P. xylostella*.

Diamondback moth, *Plutella xylostella* (L.), is the most widely distributed major pest of crucifers. The annually global costs caused by the *P. xylostella* have increased from approximately $1 billion in the 1990s[1] to $4–5 billion in recent years[2]. Due to intensive application of insecticides and the unique biological properties (such as genetic plasticity, high fecundity and short life cycle) of *P. xylostella*, this insect pest has developed resistance to 92 compounds and become the most resistant one in the world[3].

Chlorantraniliprole, a diamide insecticide, exhibited extremely high efficacy against lepidopteran insect pests since it came into the market in 2007[4]. Though the diamide insecticides functioned via a completely novel mode of action by activating the ryanodine receptor and causing hypercontraction of muscles[5,6], high levels of resistance to this newest class of insecticides in *P. xylostella* were reported in Philippines, Thailand and China after 2–3 years of extensive and repetitive application[7–9]. Most recently, an extremely high resistant *P. xylostella* field population (27,793-fold) was documented in Brazil[10]. To manage the resistance issues in *P. xylostella*, considerable efforts have been made to understand the mechanisms of the chlorantraniliprole resistance. Wang and Wu[8] found that the metabolic resistance, i.e., enhanced activities of cytochrome P450, carboxylesterase and glutathione *S*-transferases, may play a role in *P. xylostella* resistance to chlorantraniliprole. The involvement of metabolic resistance was also implicated in the Colorado potato beetle, *Leptinotarsa decemlineata*[11]. Troczka et al. (2012) identified a point mutation (G4946E) in RyR from two field populations of *P. xylostella* collected from Thailand and Philippines, respectively[7]. Subsequently, Guo et al[12] identified the same mutation in four populations of *P. xylostella* collected from Southern China, and empirically demonstrated that this mutation plays a major role in resistance to chlorantraniliprole. In this paper, three novel point mutations (E1338D, Q4594L and I4790M) in RyR were identified in a *P. xylostella* field population collected from Yunnan province, China. To be involved in the chlorantraniliprole resistance, these newly identified mutations work together with a previously reported point mutation G4946E. This is the first report that multiple mutations may be involved in diamide resistance in lepidopteran pests, and will shed light on the resistance mechanisms to this new class of insecticide.

Table 1 | Toxicity of chlorantraniliprole to different populations of *Plutella xylostella*

Population	Number	LC_{50}(95%CL)[a](mg/L)	Slope ± SE	χ^2(*df*)[b]	*P*	RR [c]	D
JA	248	0.011 (0.005–0.018)	2.02 ± 0.48	4.75 (8)	0.784	1	
TH	241	23.40 (18.34–31.28)	2.83 ± 0.53	4.18 (5)	0.523	2128	
F1(JA♀ × TH♂)	182	0.423 (0.226–0.748)	1.76 ± 0.23	17.42 (10)	0.066	38	−0.047
F1(JA♂ × TH♀)	249	0.368 (0.184–0.658)	1.83 ± 0.33	7.94 (7)	0.338	33	−0.084
F1(pooled)	431	0.399 (0.273–0.569)	1.78 ± 0.19	26.21(18)	0.125	36	−0.063
F2	438	0.613 (0.443–0.842)	1.84 ± 0.16	24.52 (16)	0.080	56	

[a]95% confidence limits.
[b]Chi-square value and degrees of freedom (df) as calculated by PoloPlus.
[c]RR = Resistance ratio.

Results

Resistance to chlorantraniliprole. Compared to the JA, the TH population showed an extremely high level resistance of 2128-fold, and the two F1 populations (JA♀ × TH♂ and JA♀ × TH♂) and F2 population showed a lower resistance of 33- to 56-fold (Table 1). The result of the genetic analysis revealed that chlorantraniliprole resistance in the TH population was autosomal control and incompletely recessive (D = −0.627) (Table 1). According to the bioassay result of F2, 0.5 mg L^{-1} and 3.0 mg L^{-1} were used as LC_{50} and LC_{90}, respectively, to treat the F2 progeny.

Identification of three novel mutations in *PxRyR*. A total of 13 cDNA fragments of *PxRyR* were amplified and sequenced directly, which covered the full coding region of the gene. Sequence alignment of TH with other *PxRyR* sequences registered in GenBank (JF926693, JF926694, JF927788, JN801028, JQ769303, JX467684) revealed that there are three novel mutations in the TH population (numbering based JN801028) (Fig. 1): (1) at position 4014, an glutamic residue (GAA) is replaced by a valine (GAT) (E1338D); (2) at position 13781, a glutamine residue (CAG) is replaced by a leucine (CTG) (Q4594L); and (3) at position 14370, an isoleucine (ATA) is replaced by a methionine (ATG) (I4790M). Moreover, a reported mutation G4946E which has been proved to be associated with the resistance against chlorantraniliprole were also found in TH population.

We further investigated the frequency of these four mutations (E1338D, Q4594L, I4790M and G4946E) in both JA and TH populations. By using PCR and direct sequencing, 45 fourth instar larvae from each of the JA and TH population were examined. One hundred percent of tested individuals in susceptible population (JA) showed the homozygous wild allele at position A4014, A13781, A14370 and G14837 and encoded the amino acid of E1338, Q4594, I4790 and G4946. While in the high resistant population (TH), 86.7% of tested individuals were homozygous mutation genotype at the same three sites (i.e. T4014, T13781 and G14370) which resulted in the substitution of corresponding amino acid, E1338D, Q4594L and I4790M (Table 2). Interestingly, no one homozygous mutation genotype was detected in the TH population and only 20% of individuals were identified as heterozygous genotype at position 4946 (Table 2).

Correlations between the four mutations and the chlorantraniliprole resistance in *P. xylostella*. To further reveal the relationship between these mutations and resistance of *P. xylostella* to chlorantraniliprole, the frequencies of each genotype of the four mutations in untreated F2 group and the survivors of the F2 treated respectively by LC_{50} (0.5 mg L^{-1}) and LC_{90} (3.0 mg L^{-1}) of chlorantraniliprole were examined. And the mortalities of the two treatments were 52.5% and 94%, respectively. In untreated F2 group, 65.7% of individuals showed homozygous wild genotype at all three novel mutation sites, and only 11.4% to 22.9% of individuals were heterozygous or homozygous mutation type (Table 2). After treated with LC_{50} and LC_{90} of chlorantraniliprole, however, the frequencies of wild type sharply decreased from 65.7% to 9.1% (E1338) and 65.7% to 12.1% (Q4594 and I4790), respectively, while the mutation type (including both heterozygous and homozygous) increased dramatically from

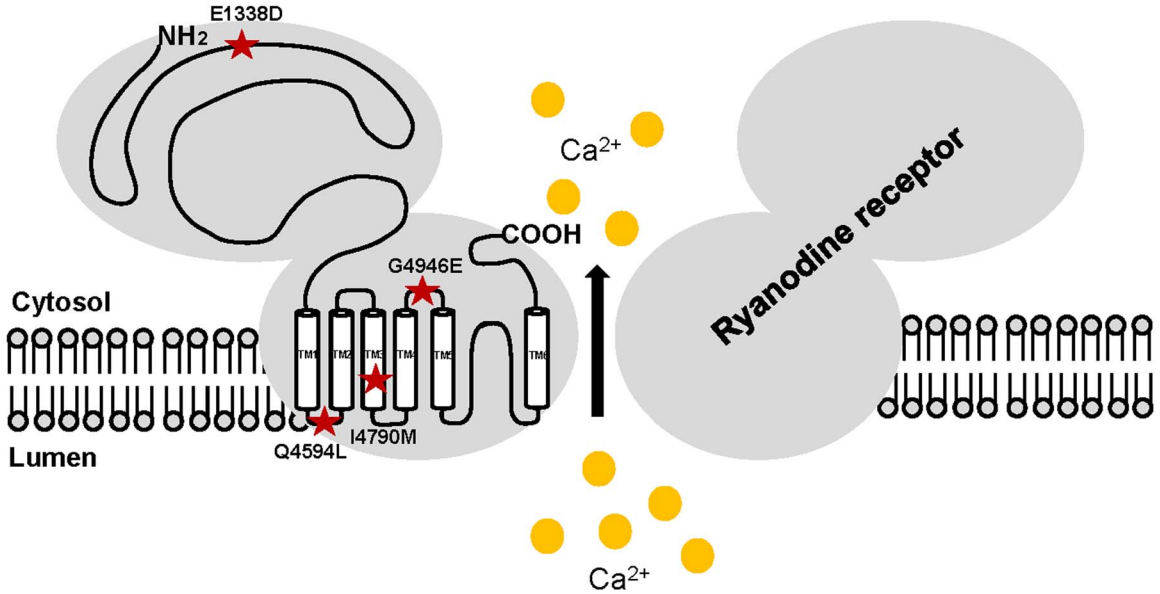

Figure 1 | Position of four mutations in *Plutella xylostella* ryanodine receptor. The position of three new mutations and reported G4946E are indicated by stars. And the positions are numbered based on the RyR sequences of the ROTH strain (accession numbers: JN801028).

Table 2 | Mutations of PxRyR and their distribution in different populations and groups

Mutation	Population (n[a])	Resistance ratio	Codons [b] (Frequency[%]) Homozygous wild	Heterozygous	Homozygous mutation
E1338D	JA (45)	1	GAA(100)	GAA/T(0)	GAT(0)
	TH (45)	2128	GAA(0)	GAA/T(13.3)	GAT(86.7)
	F2 (35)	56	GAA(65.7)	GAA/T(22.9)	GAT(11.4)
	F2-LC_{50} (31)	- [c]	GAA(29.0)	GAA/T(32.3)	GAT(38.7)
	F2-LC_{90} (33)	-	GAA(9.1)	GAA/T(24.2)	GAT(66.7)
Q4594L	JA (45)	1	CAG(100)	CA/TG(0)	CTG(0)
	TH (45)	2128	CAG(0)	CA/TG(13.3)	CTG(86.7)
	F2 (35)	56	CAG(65.7)	CA/TG(11.4)	CTG(22.9)
	F2-LC_{50} (31)	-	CAG(25.8)	CA/TG(12.9)	CTG(61.3)
	F2-LC_{90} (33)	-	CAG(12.1)	CA/TG(30.3)	CTG(57.6)
I4790M	JA (45)	1	ATA(100)	ATA/G(0)	ATG(0)
	TH (45)	2128	ATA(0)	ATA/G(13.3)	ATG(86.7)
	F2 (35)	56	ATA(65.7)	ATA/G(11.4)	ATG(22.9)
	F2-LC_{50} (31)	-	ATA(25.8)	ATA/G(12.9)	ATG(61.3)
	F2-LC_{90} (33)	-	ATA(12.1)	ATA/G(30.3)	ATG(57.6)
G4946E	JA (45)	1	GGG(100)	GG/AG(0)	GAG(0)
	TH (45)	2128	GGG(80)	GG/AG(20)	GAG(0)
	F2 (35)	56	GGG(74.3)	GG/AG(25.7)	GAG(0)
	F2-LC_{50} (31)	-	GGG(74.2)	GG/AG(25.8)	GAG(0)
	F2-LC_{90} (33)	-	GGG(51.5)	GG/AG(36.4)	GAG(12.1)

[a]: The total number of tested individuals.
[b]: Nucleotide polymorphisms are underlined.
[c]: Not available.

34.3% to 90.9% (E1338D) and 34.3% to 87.9% (Q4594 and I4790), respectively. And the frequencies of all the three novel mutations showed a positive correlation (E1338D: $R^2 = 0.984$; Q4594L and I4790M: $R^2 = 0.95$) with the corrected mortality under each concentration of chlorantraniliprole. For the G4946E, after treated with LC_{50} and LC_{90} of chlorantraniliprole, the frequencies of individuals with mutated genotype also increased but not so much, from 25.7% to 48.5%, and only 12.1% was homozygous mutation type in the LC_{90} treated group (Table 2).

To understand whether the different mutation combinations confer resistance to chlorantraniliprole in the *P. xylostella*, the frequencies of all detected mutation combinations were analyzed in F2 as well as the LC_{50} and LC_{90} treatment group. There were 12 mutation combinations in all the populations and groups. And it is noteworthy that the mutations Q4594L and I4790M are completely linked in all individuals examined (Table 3). In addition, the number of mutation combinations increased dramatically in the LC_{50} (8 combinations) and LC_{90} (9 combinations) of chlorantraniliprole treatment group compared to that of the untreated F2 population (5 combinations). In the untreated F2 population, the mutation combination 2 (three homozygous wild genotypes and one heterozygous mutation; A^{4014}, A^{13781}, A^{14370}, G/A^{14837}) was the predominant mutation combination. While in the LC_{50} and LC_{90} treatment groups mutation combination 3 (three homozygous mutations

Table 3 | Combinations of different genotype in the different treatment group

Mutation combination	Frequency % CK	LC_{50}	LC_{90}	Genotypes at each mutation site E1332D A to T	Q4547L A to T	I4743M A to G	G4946E G to A
1	40.0	16.1	/	A	A	A	G
2	25.7	9.7	6.1	A	A	A	G/A
3	8.6	25.8	24.2	T	T	G	G
4	14.3	22.6	21.2	A/T	T	G	G
5	8.6	6.5	/	A/T	A/T	A/G	G
6	2.9	/	6.1	T	A/T	A/G	G
7		6.5	3.0	T	T	G	G/A
8		6.5	24.2	T	A/T	A/G	G/A
9		3.2	/	A/T	T	G	G/A
10		3.2	/	A	T	G	G
11			3.0	A	A	A	A
12			3.0	A/T	A	A	G/A
13			9.1	T	T	G	A
N[a]	35	31	33				

[a]: Individuals tested.

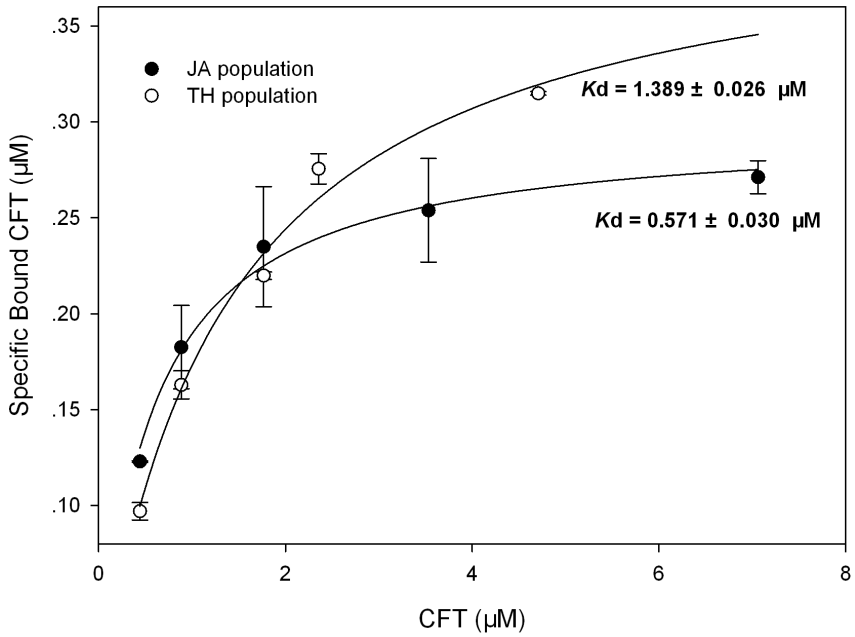

Figure 2 | **Equilibrium binding of chlorantraniliprole fluorescent tracer to membrane protein of *P. xylostella*.**

and one homozygous wild genotype; T^{4014}, T^{13781}, G^{14370}, G^{14837}) became the predominant mutation combination. It is worth noting that three new mutation combinations appeared specifically in the LC_{90} treatment group with a total frequency of 15.1%, and more than half of them were mutation combination 13 (four homozygous mutations, 9.1%).

Affinity of PxRyR to chlorantraniliprole in TH population. To understand the effect of multiple mutations on the binding affinity of PxRyR to the diamide insecticides, the binding affinities of chlorantraniliprole fluorescent tracer (CFT) to PxRyR prepared from both the susceptible and TH populations were determined. As shown in Fig. 2, the *K*d value in TH population was 1.389 ± 0.026, which was 2.4-time of that in the susceptible population (*K*d = 0.571 ± 0.030) (t = 29.358, P = 0.0012).

Detection of the mutations by allele-specific PCR (AS-PCR). To identify these mutations, three groups of allele-specific PCR primers were designed to examine the genotypes of E1338D, Q4594L and G4946E (mutation I4790M was not considered because it's completely linked with the Q4594L). The genotypes of these three mutation sites in 36 fourth instars from both JA and TH populations were tested individually by using the AS-PCR and direct sequencing. The results showed that the genotypes of the three mutations can be detected accurately by using the AS-PCR with a rate of accuracy 98% (Fig. 3).

Discussion

The global market of diamides has increased substantially since 2007 and this new class of insecticides has become one of the primary chemical control agents against the lepidopteran pests due to their biological, ecological and toxicological attributes[13]. However, intensive and repetitive use of the diamides have led to the rapid development of resistance in both *P. xylostella* and *S. exigua*[7,8,10,14].

Most recently, a point mutation (G4946E) in *P. xylostella* RyR has been identified and empirically confirmed to confer high level of resistance to chlorantraniliprole in field populations of Thailand,

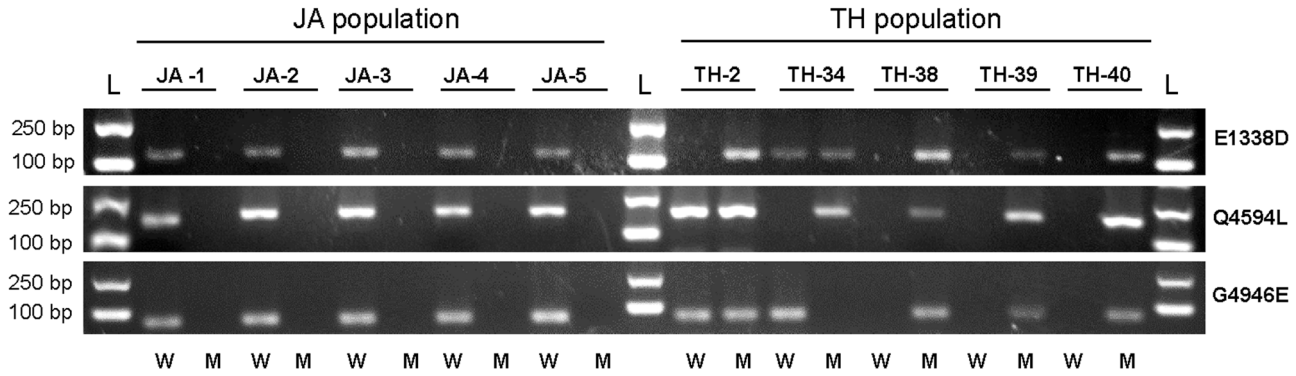

Figure 3 | **Diagnosis of different genotypes of the mutations using allele-specific PCR.** Five individual forth instar larvae from both JA and TH populations were detected using both susceptible-specific (W) and resistant-specific (M) primers, respectively. Samples from the JA population are all homozygous wild type at all three mutation sites, while the sample TH-2 are heterozygous at Q4594L and G4946E, and TH-34 are heterozygous at E1338D, homozygous mutation genotype at Q4594L and homozygous wild genotype at G4946E. TH-38, 39 and 40 are all homozygous mutation genotypes at all three sites. The products of PCR were examined on a 2.5% agarose gel and stained with ethidium bromide after electrophoresis. The length of the products for E1338D, Q4594L and G4946E detection are 116 bp, 168 bp and 71 bp, respectively. L represents the DL 2000 DNA Marker (Takara Biotechnology, Dalian, China).

Table 4 | Primers used for frequency examination and allele-specific PCR

No.	name	Primer sequence	Length of products (bp)
Sequencing primer			
1	F-3679	GAT ACG AGC CAT TCT GTG T	530 (containing mutation E1338D)
	R-4208	TGC CAT TCT GTT GAC CTG	
2	F-13512	CCC ACG GAA GAG GAG AAA	791 (containing mutation Q4594L and I4790M)
	R-14302	TCC AGT TTA CGG GCT ATC T	
3	F-14596	ACA ACT CGT TCC TAT ACT CTC	152 (containing mutation G4946E)
	R-14747	TGT TTC CCG TTA TGC GTG AC	
Allele-specific primer			
4	F-3979-S	ATG AAG GAG GCA GCT **C**AA	116 (for E1338D genotyping)
	F-3979-R	ATG AAG GAG GCA GCG **C**AT	
	R-4361	TCC TCT TGA CCA TCA TCA TAG T	
5	F-13620-S	GGA AGA TAA TGG ACA AGT **T**CA	168 (for Q4594L genotyping)
	F-13620-R	GGA AGA TAA TGG ACA AGT **T**CT	
	R-13787	GCT TGC TTC TTA GCT TGT TC	
6	F-14677-S	TGT TGG ACG TGG CTG T**A**G G	71 (for G4946E genotyping)
	F-14677-R	TGT TGG ACG TGG CTG T**A**G A	
	R-14747	TGT TTC CCG TTA TGC GTG AC	

The differences of susceptible and resistant allele specific nucleotides were underlined, and the mismatched nucleotides were marked in bold.

Philippines and China, respectively[7,12]. This mutation has been identified in different populations from at least three countries in East Asia, suggesting that the mutation G4946E may be a universal mechanism for diamide resistance in *P. xylostella*. In this paper, however, only 20% of the individuals possessed this mutation in a highly resistant (>2000-fold) *P. xylostella* field populationcollected in Yunnan province, China. The full length *PxRyR* cDNA was cloned and sequenced and three novel mutations (E1338D, Q4594L and I4790M) were identified in this Yunnan population (Fig. 1). Although lack of a complete crystal structure data of a RyR protein as a reference, the physical location of these mutations suggest an potential impact on the binding of diamides to PxRyR. First, the mutations located at critical motifs in PxRyR. The Q4594L is located within a long loop of predicted transmembrane domain TM1 and TM2, and more importantly, it is near a diamide-sensitive region (with a length of 46 amino acids identified in *Drosophila menlanogaster*) by Tao et al.[15], and the mutation I4790M is located at TM3, which is important for the formation of the calcium channel pore. Although the mutation E1338D is located near the N-terminal of PxRyR and not in or near reported putative functional regions, we cannot exclude the possibility that it may be involved in the resistance because N-terminus region can be a structural requirement for diamides-induced activation of the RyR[16]. Second, a multiple amino acid alignment of all sequenced RyRs from 31 insect species revealed that the amino acids at the three mutation sites are highly conserved (100% identity) in all lepidopterans (10), but varied in the remaining ones (21)(Supplementary information Fig. S1). For example, at position 4594, it is glutamine (Q) in Lepidoptera, while in the remaining 21 non-lepidopterans it is lysine (K), arginine (R), alanine (A), and other different amino acid substitutions, respectively. While at position 4790, it is isoleucine in Lepidoptera but methionine (M, same as the mutated one in *P. xylostella*) in all other 21 non-lepidopterans. The highly conserved in Lepidoptera but highly variable nature in non-lepidopterans at the three mutation sites likely contributes to the high selectivity of chlorantraniliprole between the lepidopterans and the other insect orders.

To link these mutations with resistance[17], the frequencies of these mutations in different populations and groups were analyzed. And this method have been used successfully in analyze different mutations in many pests, including *Haematobia irritans*[18], *Bemisia argentifolii*[19], *Tetranychus urticae*[20,21], *Trialeurodes vaporariorum*[22], *Culex quinquefasciatus*[23] and *P. xylostella*[12]. To further reveal the relationships between these mutations and chlorantraniliprole resistance, the frequency of these mutations or different mutation combinations were examined.

The results showed that the frequency of each of the three new mutations in TH population was 100%, but none was detected in the JA population. In the highly heterozygous F2 population, the frequency for each of these mutations was only 34.3%, however, after treated with chlorantraniliprole (LC_{90}), the frequency of all three mutations in the survived individuals were elevated to approximately 90% the treated group. And the changes of the mutation frequencies were highly correlated to the corrected mortalities of LC_{50} and LC_{90} treatments ($R^2 \geq 0.95$). These results strongly suggested that the three mutations are, at least, partially involved in the chlorantraniliprole resistance in *P. xylostella*. For a recently reported mutation G4946E, which plays a major role in chlorantraniliprole resistance in several other field *P. xylostella* populations[7,12], it accounted only 20% of mutated individuals in the TH population, suggesting a reduced role of G4946E in chlorantraniliprole resistance in this population.

It is noteworthy that all four mutations appeared in various of combinations in TH and F2 populations as well as the survivors of the F2 generations treated respectively with LC_{50} and LC_{90} of chlorantraniliprole. And with the increase of the treatment concentration, the number of combinations increased. Especially in the survivors of LC_{90} treated group, 36.3% individuals possessed all four mutations (Table 3, combinations 7, 8, 9 and 13). These results strongly suggested that the combination of mutation rather than a single mutation may play important role in the chlorantraniliprole resistance in this population. In insects, the concomitant mutations in sodium channel confer high knockdown resistance (*super-kdr* resistance) to DDT or pyrethroid insecticides has been shown in *Musca domestica* (L1014F + M918T)[24], *Haematobia irritans* (L1014F + M918T)[25] and *P. xylostella* (L1014F + T929I)[26]. Recently, Li et al.[23] reported that the co-existence of nine mutations (3 nonsynonymous and 6 synonymous mutations) in sodium channel was indeed correlated with the high level of permethrin resistance in *Culex quinquefasciatus*. It is notable that there were 6% individuals survived in LC_{90} treatment group. Because one of the original parental populations was collected from the field with complex resistance mechanisms, the resistant mechanism of these individuals may involve detoxifying enzymes, such as P450.

In our previous study[12], we have found that the increased *K*d value of PxRyR to the CFT was correlated to the chlorantranilprole resistance. TH population also had a significantly higher *K*d value than

that of the susceptible JA population (Fig. 2), indicating that the resistant *P. xylostella* possessed decreased affinity to the chlorantraniliprole than the susceptible one. Therefore, multiple mutations in the TH population may result in the reduced binding affinity of PxRyR to the chlorantraniliprole, and lead to the chlorantraniliprole resistance.

The combined results suggest the high level of chlorantraniliprole resistance in Yunnan and Guangdong populations may evolve independently. Geographical isolation is a potential cause contributing to this difference. In conclusion, we have identified three novel and one known mutation in PxRyR from a field resistant *P. xylostella* population and experimentally demonstrated that the co-existence of these mutations might be responsible for the high level of resistance to the chlorantraniliprole through reduced binding affinity. An AS-PCR based mutation diagnosis method was developed to genotype the field samples. Although the quantitative contribution of these mutations and their combinations to the sensitivity of *P. xylostella* to diamides remains to be determined, our results demonstrated that multiple mutations can work collaboratively to confer resistance and this work provides the basis for the mechanistic understanding of diamide resistance.

Methods

Insects. The susceptible population of *P. xylostella* (JA) was kindly provided by Professor Tadashi Miyata in 2010, which has been maintained in Nagoya University, Japan for more than 60 years. The resistant population (TH) was collected from field in Tonghai city, Yunnan Province, in southwestern China. All stages of *P. xylostella* were reared in our laboratory using vermiculite cultured radish (*Raphanus sativus* L.) seedlings and maintained at 27 ± 1°C, 40–60% relative humidity (RH) and a 16 h light: 8 h dark photoperiod.

Bioassays. The chlorantraniliprole was of technical grade (95%) and supplied by Dupont Agricultural Chemicals Ltd., Shanghai, China. Bioassays were conducted using the leaf-dip method according to Liang et al.[27] and Guo et al.[28]. Chinese cabbage leaves measuring 6 × 6 cm were immersed for 10 s in various concentrations of chlorantraniliprole prepared with distilled water containing 1 g L^{-1} TritonX-100. The leaves were allowed to air dry and were then placed individually into a culture dish with a filter paper. A total of 10–15 two-day old third-instar larvae were introduced into each dish, and three replications were conducted. Five to seven concentrations of chlorantraniliprole and one control (leaves treated with distilled water containing 1 g L^{-1} Triton X-100) were tested in each bioassay. Mortality was assessed at 96 h after treatment. Individuals that did not move when pushed gently with a brush were scored as dead. Concentration–mortality data were analyzed by probit analysis using PoloPlus 2.0 (LeOra Software, Petaluma, CA). The degree of dominance (D) of chlorantraniliprole resistance was calculated according to Stone's method[29]. The D value stands for that the resistance is controlled by completely dominant gene (D = 1), semidominant gene (D = 0), completely recessive gene (D = −1), incompletely dominant gene (0 < D < 1) or incompletely recessive gene (−1 < D < 0).

RNA extraction, cDNA synthesis and sequencing of *Plutella xylostella RyR* (*PxRyR*). Total RNA was extracted from pools of 10 fourth instars using TRIzol kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. First strand cDNA was synthesized using Thermo Scientific Verso™ cDNA Synthesis Kit (Thermo). Thirteen pairs of primers (Supplementary information Table S1) for amplifying the full length of *PxRyR* were designed based on a *P. xylostella* RyR sequence (GenBank accession JF926693) using Primer Premier V.6.0 (Premier Biosoft International, Palo Alto, CA). All PCR products were purified and sequenced directly by Invitrogen (Shanghai, China). Alignment of sequencing results was analyzed using DNAman v 6.0 Software (Lynnon Biosoft, Quebec, Canada).

Crosses and linkage analysis. To further identify the linkage between mutations and chlorantraniliprole resistance in *P. xylostella*, the chlorantraniliprole susceptible population (JA) and resistant population (TH) were reciprocally crossed (JA♀ × TH♂ and JA♂ × TH♀) to produce hybrid F1 populations by allowing 50 virgin female adults from one population to mate freely with 50 adult males from the other population. Because their LC_{50} values showed no difference, the F1 (JA♀ × TH♂) and F1 (JA♂ × TH♀) were pooled together and allowed to mate randomly to get a hybrid F2 population.

The resistance level of F2 population to the chlorantraniliprole was tested by using the leaf-dip method as described above and the LC_{50} and LC_{90} values were calculated. Then three cDNA fragments were amplified using the primer 1–3, and then sequenced directly to identify the genotype of the four mutation sites individually in the fourth instar larvae from F2 as well as the F2 treated by LC_{50} (0.5 mg L^{-1}) and LC_{90} (3.0 mg L^{-1}) of chlorantraniliprole, respectively. The fragments amplified by gene-specific primer 1 and 3 (Table 4) contains position 1338 and 4946, respectively, while the fragment amplified by primer 2 (Table 4) contains mutation sites 4594 and 4790. A total of 30 to 50 individuals from each group were examined.

Membrane preparation and fluorescence polarization binding assays. The membrane protein was prepared according to the method of Scott-Ward *et al.*[30] and Cordova *et al.*[5]. Briefly, 1 g of fresh fourth instar larvae were homogenized in 9 mL of ice cold buffer A (50 mM Tris-HCl, pH 7.4, containing 1 μg mL^{-1} of aprotinin, pepstatin and leupeptin, respectively). The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The resulting supernatant was passed through five layers of filter paper, and the filtrate was collected in a 10-mL centrifuge tube and centrifuged at 10 000 × *g* for 20 min. Then the collected supernatant was centrifuged for 60 min at 100 000 × *g* and the resulting pellet was re-suspended in a minimum volume (3–5 mL) of buffer B (1.5 M KCl, 300 mM sucrose, 0.5 mM $CaCl_2$, 20 mM Tris-HCl, pH8.0, containing 1 μg mL^{-1} of aprotinin, pepstatin and leupeptin, respectively). The samples were then snap frozen in liquid nitrogen and stored at −80°C until used. The protein concentration was determined by Bradford assay using BSA as a standard[31].

The fluorescent polarization (FP) binding assay described by Dandliker *et al.*[32] and Hatzidakis *et al.*[33] was adopted to determine the binding affinity of the chlorantraniliprole fluorescent tracer[34] to the prepared membranes. First, the optimum concentration of the membrane protein for the reaction was determined. The stock membrane solution prepared was diluted by 1, 2, 4, 8, 16, 32, 64, 128 and 256 times using buffer A. Then 50 μL of membrane solution was mixed together with 50 μL of CFT (0.706 μM) and 50 μL of buffer A. The mixture was incubated at 25°C for 20 min and then transferred into a 200 μL quartz tube to measure the FP intensities using Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Inc., California) equipped with an FP apparatus. The excitation and emission wavelengths were set at 396 and 475 nm, respectively, and the width for both excitation and emission wavelength is 10 nm. Each concentration of membrane protein was measured three times. The dilution factors of the membrane protein were logarithm transformed and then plotted against the corresponding FP intensity. Then the optimum membrane concentration that caused half of the maximum FP intensity was determined by using the dose-response analysis of the Origin software (V7.0, OriginLab Corporation).

To determine the dissociation constant (*K*d) values of the CFT binding to the prepared membrane protein, the CFT was diluted with buffer A to get seven concentrations (7.2, 4.8, 3.6, 2.4, 1.8, 0.90 and 0.45 μM), then 50 μL of diluted CFT was mixed together with 50 μL of the optimum concentration of membrane protein and 50 μL of buffer A. The FP intensity of the mixture was measured as described earlier. Three replicates were conducted for each concentration of CFT.

Then the FP intensities and the logarithm transformed membrane protein concentrations were subjected to the ligand binding module of SigmaPlot 10.0 (Systat Software Inc., San Jose, California) to calculate the theoretical minimum FP intensity (P_{min}) and the maximum FP intensity (P_{max}). The ratio of bound ligand (*B*) and free ligand (*F*) was calculated using the equation: $Xi = B/F = (P - P_{min})/(P_{max} - P)$, of which *P* is the observed FP intensity. For the total ligand concentration $T = B + F$, therefore $B = Xi \times T/(Xi + 1)$. Then the calculated *B* values and the concentrations of membrane protein were subjected to ligand binding analysis using the SigmaPlot 10.0 to calculate the *K*d value.

Allele-specific PCR diagnostic assays for *PxRyR* genotyping. Allele-specific polymerase chain reaction (AS-PCR) was employed to detect the genotypes of the mutation E1338D, Q4594L and G4946E but not the I4790M, for it is completely linked to the mutation Q4594L. To increase the specificity of the primers, a mismatched nucleotide was introduced into the third position from the 3′ end of the forward primer (Table 4, primer 4–6). The reaction system of PCR comprised of 2.5 μL 10× Ex Taq buffer, 2 μL 2.5 μM dNTP mixture, 0.5 μL 10 mM forward primer, 0.5 μL 10 mM reverse primer, 1.5 U of Ex Taq DNA polymerase (Takara Biotechnology, Dalian, China), 1 μL of template cDNA and then made up to a final volume of 25 μL with double distilled H_2O. The PCR cycling was performed with an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of amplification: 94°C for 20 s, 60°C (55°C for primer 6) for 20 s and 72°C for 20 s. The final extension was performed at 72°C for 2 min. PCR products were visualized on a 2.5% agarose gels in 1× TBE buffer, loaded with 5 μL of reaction mix and stained with ethidium bromide after electrophoresis.

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Author contributions

Conceived and designed the experiments: P.L. and L.G. Performed the experiments: L.G. Analyzed the data: P.L. and X.Z. Contributed reagents/materials: P.L. and X.G. Wrote the paper: P.L., L.G. and X.Z.

Additional information

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